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[Studium listové epidermis mikroreliéfovou metodou]

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STUDY OF THE EPIDERMIS OF THE LEAF BY THE MICRORELIEF METHOD

Jaroslav Pazourek

During microscopic observation of the plant surface, we often use the so-called microrelief methods. They consist in the addition of a thin layer of fast hardening transparent substances, on which the surface relief is perfectly imprinted. After complete hardening, the thin layer is removed and observed under the microscope.

This method was already proposed in 1891 by H. Berry, but it was applied to the epidermis imprint only by L. Buscalioni and G. Pollacci [1902]. At first, it served for the study of transpiration, and the collodion used in the preparation of the films would become clouded in regions of higher humidity. Later, the method was used in the same way for relatively more detailed work [e.g., K. Rudolph, 1925]. Soon after, various authors began to use this method, especially for microscopic observation of fossil surfaces [see the literature: F. L. Long and F. E. Clements, 1934], and later, for the observation of living plants [D. Peterson, 1929; V. Neuwirth, 1930; E. Ashby, 1932, A. Wallach, 1939; et al].

J. Wolf [1938a, 1938b, 1939, 1954] modified the method for various purposes of medical histology. Wolf's modification consists in taking imprints using cellophane ribbon. It permits not only the

convenient removal of the diaphragm, whose delicateness often caused difficulties, especially in the case of imprints of plant epidermis where the picture of a large surface must be considered, but also the rapid treatment of large amounts of material. We commonly use it for imprints of plant epidermis [S. Prát, 1952; Z. Pazourková and J. Pazourek, 1960; B. Němec et al., 1962].

Substances used for the preparation of imprints

Membrane substances on which the microrelief of the epidermis surface is imprinted include the abovementioned collodian, celloidin, celluloid, dissolved in acetone, ether, alcohol-ether, etc., as well as a strongly concentrated liquid of unburnable photographic film (without emulsion) dissolved in ethyl acetate [J. Václavík, 1955]. F. L. Long and F. E. Clements [1934] have tested over 150 substances and liquids in order to find the most convenient one for general as well as special purposes. H. Wenzel (1939) also experimented with a series of substances, soluble and insoluble in water. Both works contain valuable findings which are important for the application of the microrelief method.

During my work, I have tried to find substances which would be most convenient in the application of the microrelief method, i.e., which would eliminate most of the difficulties (e.g., the clouding of membranes), and which would be easy to obtain. I found three suitable substances.

Colorless fingernail lacquer. It is the most common, the most easily available, and the most widely used. Most frequently, I used the brand Synfleur 0 and Simpson 0, but it seems that there is little difference between the products. It is necessary, however, to adjust the density of the solution; either we dissolve it in acetone, or we leave it to thicken.

Lacquer for panels (methyl ester methacrylic acid) is sold in small bottles of ten ml (produced by Dental Co.). Although it is

possible to dilute it with chloroform, it is better to use it in the original state, and to prevent its thickening and later glutination by careful closing. It thickens very fast, and for that reason it is necessary to work unusually fast: this quality is a guarantee of the proper imprint of the surface. It proved valuable for the study of the state of air holes and in research on water plants epidermis [I. Möllerová, 1962].

Polyvinyl acetate in ethanol. In regard to Lloyd's method, it can be assumed that ethanol changes the state of the epidermis the least. For that reason, I considered the use of ethanol as the solvant in the microrelief method most convenient. As substances for the membrane preparation, I used polyvinyl acetate, dissolved in ethanol. It is, to be sure, not possible to use water-free ethanol, but we obtain good pictures with it. In 96% ethanol, the polyvinyl acetate dissolves very slowly and relatively little; it is better to use the concentrated ethanol. Adding a small quantity of acetone augments the solubility. If we introduce it in a thicker layer, it will become clouded.

Possibilities for the application of the microrelief method in the study of the epidermis

In botany, the microrelief method is most often applied in the study of the state of air holes. It is also permissible to use it for anatomical work, for observation of the epidermis structure, forms of epidermal cells, analysis of air holes, etc. It is especially convenient in quantitative anatomical work, for identification of size, area of epidermal cells, length of anticline per unit of surface, number of air holes per unit of surface, length of air holes (e.g., study of polyploides), etc.

J. Wolf [1954] describes the appearance of the picture in the microscope using this method. In order to have a good and usable picture, it is necessary that there be a sufficiently clear relief on the surface, i.e., sufficient elevation differences. In the study

of epidermal cells, it is first of all a question of distinguishing the locations of the anticlines. It is necessary to understand that it is really not a question of the representation of cellular membranes which pass vertically to the surface, but a question of relief imprint.

Good pictures were obtained where the epidermal cells were convex and the external cellular membranes were sharply inclined toward locations from which the anticlines started. This is true, for example, of the upper and lower epidermis of the phaseolus vulgaris (Table XIX, 1, 2). In order that the cells be clearly distinguished, it is necessary to adjust the lighting. In case of serial work, for drawing or for microphotography, it is best to close the diaphragm, in certain cases, even to lower the condensor setting. Even when working with convex cells that are the most convenient for the microrelief method, the reliefs will still cause heavy obscuring in the picture, so that it is difficult to find the anticlines (Table XIX, 3); only in case of heavier obscuring do we receive a good picture. Unevenness of external cellular membranes, deepening and rising in relief, however, cause effects similar to what occurs over anticlines. It is necessary, therefore, to be aware of this fact and to correctly evaluate the demarcation of the cells. Mistakes can occur, especially in the case of cells near air holes (Table XIX, 4).

In the case of strong obscuring, we will lose our ability to differentiate, but this is not the point; the aim is to obtain clear bright anticlines on a dark background (Table XIX, 5).

The form of the cell surface, i.e., the relief of the epidermis, naturally strongly influences the quality of the preparation and of the microscopic picture. In detailed work, in which a precise drawing of the anticlines is needed, it is important to obtain clear lines for the thinnest and the most precise anticlines possible. In this case, often the flattest cells with relatively shallow and steeper grooves over the anticlines give better results than the more convex cells. In the first case, the lines are thinner and sharper than in the second case with thicker lines which are not

sufficiently distinct and limited, because here numerous reflections cause disturbances. Here, it is necessary to choose the most convenient opening of the diaphragm, and not to shade too much (Table XIX, 6, 7). The smooth and shiny leaves have insignificant differences in the depth of the epidermis relief; in these cases, we can recognize the cells only in case of very strong obscuring, and many times the lines separating the cells are still thick and not distinct (Table XIX, 8). In case of shading, however, various impurities appear — for example, inequalities in the ribbon which is used to photograph the membrane. We can, however, eliminate them [J. Wolf, 1954], but usually there is no need for this. It does not especially matter, because in ordinary work it is not important either for minor or major enlargements. The enlargement itself, especially the choice of various objectives, does not have any influence on the quality of the picture. Perhaps only a smaller depth of field prevents one from making the whole field of vision sharp, because even a very well stretched ribbon still has moderate inequalities. This difficulty complicates the work, especially when we prepare a series of photographs. If the epidermal cells protrude more than in the case of small enlargement (weaker objective), the cells will be depicted with much more surface. This is convenient in cases of descriptive work; if it is only a question of exposure of the anticlines for quantitative work, the clear centers of cells cause disturbances (Table XIX, 9). In that case, a higher quality picture is obtained by using a clearer, high-magnification objective (Table XX, 1).

The method also has its limitations.

In some cases it can be used only with great difficulty, or not at all. Thus, in the case of smooth and shiny leaves where the surface is covered by a relatively thick cuticle, which fills out the inequalities of the epidermis surface, it is very difficult or impossible to identify the passage of the anticlines. At the same time, the deeper parts appear over the anticlines, as well as other deep regions. Therefore, the complete picture obtained is a mixture of variously clear and not distinctly limited lines (Table XX, 2). The wrinkles on the external diaphragms of the cellular cuticle

frequently cause difficulties. If they are dense and pass through several cells, then it is impossible to recognize and to demarcate individual cells (Table XX, 3). This method, however, could be applied in the wrinkle passage observation. Whether we can also observe the wrinkles in the cells is determined not only by the number and size of wrinkles, but also by the protuberances of the cells (Table XX, 4).

The covering of trichomes presents us with another difficulty. Usually, however, this does not present too great a problem, because we also find many cells among the trichomes, which are depicted very well by the microrelief method. If the covering is felt-like and too dense, it is simpler here to scrape it off with a blade. The trichomes frequently become suffused in the substance, from which we make the membrane, and while peeling it, we tear it off. If we prepare a new imprint from the same region, it is of much better quality (thereby impurities are also eliminated). This repeated peeling of the microrelief membrane is, moreover, the only way to eliminate the wax released on the surface of some leaves, which completely prevents observation (Table XX, 5, 6). It is, however, necessary to prepare the second imprint as soon as possible after the first.

The differences between membranes prepared from different substances are negligible (Table XX, 7, 8, 9). It appears, however, that panel lacquer, which hardens fastest, is the most convenient. The solid material content in the solution is the largest and, therefore, the relief is best preserved.

Influence of temperature and humidity on the proportions of depicted structures

During my work, I observed, after a certain time, that the ribbon, in the places where the membrane is underneath, is no longer as straight as immediately after the preparation is made. Because this waviness can be observed only where the membrane is attached, or in its immediate vicinity, it is possible to establish that this

phenomenon is influenced by the material from which we prepared the imprint. The concavity itself, however, is probably caused by a certain distention of the ribbon. After several peelings-off of the ribbon, due to its distention and its reattachment, there appear on the membrane small crevices, which indicate that the membrane itself did not change its proportions. The crevices, however, are of negligible dimensions.

It is completely unimportant whether the extension and expansion is caused by the membrane or by the ribbon. It is more important to know the consequences, and whether it can affect the quantitative results obtained by this method.

It is self-evident that the greatest difficulties appear during microphotography. But even during the drawing, we must constantly sharpen the image. This is not only unpleasant, but this can lead to various inaccuracies in quantitative anatomical work, because the surface of the cells depends on whether its relief imprint is placed obliquely. We determine, therefore, not the real surface, but its projection into the plane which is oblique with respect to the optical axis of the microscope. Thus, we obtain lower values than really are on the imprint. This can be simply eliminated by tearing off one side of the ribbon from the slide, and reattaching it after careful extension. The question then arises of whether (again, in regard to the elasticity of the ribbon and, finally, also of the membrane itself) by the extension of the structure we do not stretch it to such an extent that we obtain higher values.

I have observed in several experiments the possibility of all the abovementioned changes. As an example, there is the experiment with microreliefs of the underside of a Magnolia obavata leaf. The preparation was made by the common method, using panel lacquer. During the experiment, I drew two cells at different time intervals with an Abbe drawing instrument, at different temperatures and relative humidity, which were in the laboratory at that time. After finishing the experiment, I measured the cells with a planimeter and

converted the values into μ^2. The ribbon was redistended during the entire experiment; only during the last measurement did I detach it, distend it, and again fasten it.

Date	Hour	°C	Rel. humid.	Cell area, μ^2	
				Cell I	Cell II
11. V.	17,15	24,5	47	1084	1601
	19,20	21,5	47	1005	1527
12. V.	8,30	17,5	55	1042	1521
13. V.	9,00	20,5	59	1050	1513
	11,00	22,0	63	1060	1567
	14,00	25,0	56	1031	1538
	17,30	25,0	51	1047	1479
	20,30	24,0	46	1048	1528
15. V.	12,00	27,0	44	1031	1528
16. V.	9,30	20,5	51	991	1500
				(before switching off)	
	9,35	20,5	51	1087	1588
				(after switching off)	

Variations in values can be caused by different positions of the cell imprints in the case of different distensions of the ribbon, or by random errors during the drawing and measuring. Certainly, one can see that the values obtained from the distended membrane are greater than the previous ones, and which correspond more closely to the initial ones.

Changes in the preparation and the enlargement of the ribbon in the region where the membrane is attached could be caused or at least influenced by external effects, i.e., by temperature and by relative humidity. This circumstance could also have an influence on possible differences during measurement and could produce inaccuracies.

To determine whether some dependences exist and whether it is necessary to correct the measured values by those factors, I measured the two cells under different conditions of temperature and relative humidity in the same way as in the previous experiment.

In regard to the results of previous experiments, I turned off the ribbon with the membrane before each drawing. In order to

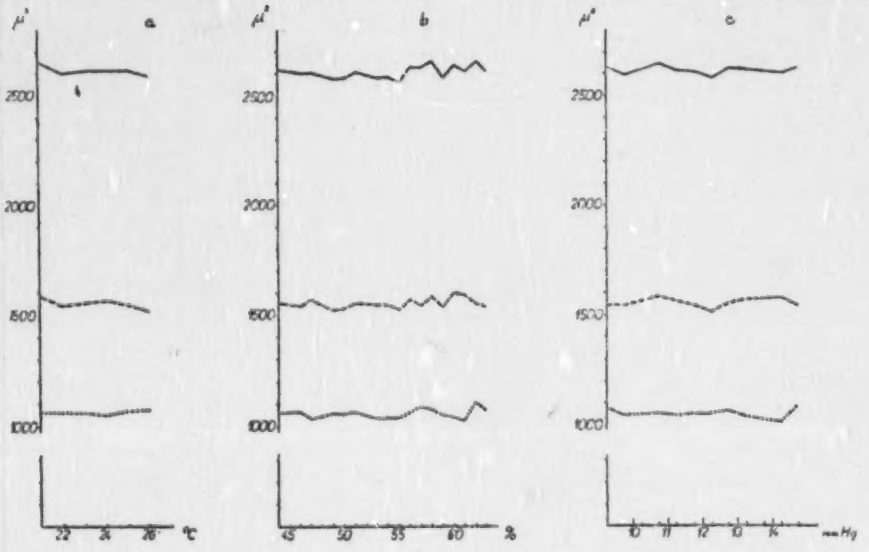

Figure 1. Graphic representation of fluctuations of the measured area of two Magnolia obovata epidermis cells,(at various a) temperature, b) relative air humidity, c) absolute air humidity; ..... — first cell; ----- — second cell, ——— — area of the two cells

eliminate the mistakes during the drawing, I always drew the cells four times, and I measured it with the planimeter two times. The experiment lasted from May 17 to May 28, 1961. Thus, I found 50 values in all, which I listed according to: a) temperature; b) relative humidity; c) absolute humidity. I set forth the results in Figure 1. The values shown here are the average of the values measured on various days, always at the same temperature and humidity.

In exactly the same way, I drew and measured the cells of Acer platanoides. The results of the same experiment are summarized in the graph in Figure 2.

As can be seen from the graphs shown, neither the temperature nor the relative and absolute humidity have any influence on the changes in the size of the measured area. Fluctuations in the values are evident, however, but they do not manifest any regularity, irrespective of whether we observe the area of independent cells or

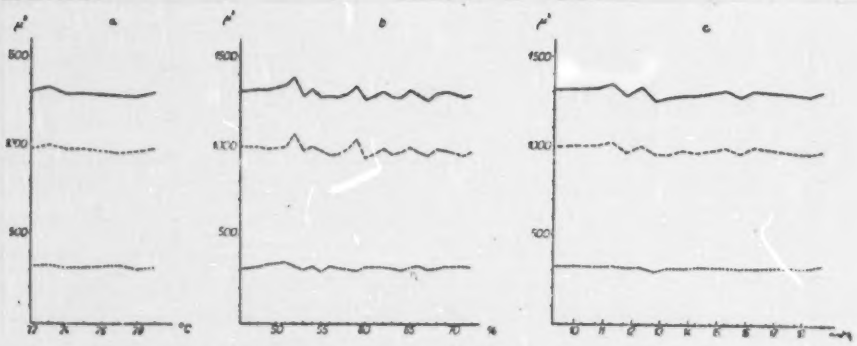

Figure 2. Graphic representation of fluctuations in the measured area of two epidermal cells of Acer platanoides at different a) temperature; b) relative humidity; c) absolute humidity; ..... — first cell; ----- — second cell; ——— — total area of both cells

their total area. The fluctuations are caused, above all, by the errors that occur during drawing, e.g., measurements. Generally there are smaller errors in cases of larger areas (e.g., the cells of of Magnolia) than in cases of smaller areas (e.g., the cells of Acer), if we express these errors in percentages. Thus, for example, in the case of the total area of both cells of Magnolia, the greatest variations are from 1 to 2% of the average surface area, but with Acer platanoides, there is a large deviation from the average at the relative humidity of 52% (Figure 3b)* and of 8.9%. For this reason, it is better to draw smaller cells with a higher enlargement; occasional errors which arise during drawing are considerably lower. In general, the results of both experiments show that neither temperature nor humidity influences microrelief experiments to such an extent that the final results are affected.

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*Translator's note: The reference should be to Figure 2b.

TABLE XIX. MICRORELIEF IMPRINTS PREPARED WITH THE HELP OF PANEL LACQUER

1. Phaseolus vulgaris, upper epidermis, small enlargement.
2. Phaseolus vulgaris, lower epidermis, small enlargement.
3. Salvia splendens, lower epidermis, large enlargement, normal lighting.
4. Salvia splendens, lower epidermis, large enlargement, strong shading.
5. Deutzia crenata, upper epidermis, large enlargement.
6. Persica vulgaris, upper epidermis, large enlargement.
7. Persica vulgaris, lower epidermis, large enlargement.
8. Parthenocissus tricuspidata, upper epidermis, small enlargement.
9. Robinia pseudo-acacia, upper epidermis, small enlargement.

TABLE XX. MICRORELIEF IMPRINTS PREPARED WITH THE HELP OF PANEL LACQUERS

1. Robinia pseudo-acacia, upper epidermis, large enlargement.
2. Mahonia aquifolium, upper epidermis, large enlargement.
3. Cerasus vulgaris, lower epidermis, small enlargement.
4. Deutzia crenata, lower epidermis, small enlargement.
5. Salix alba, lower epidermis, small enlargement, imprint I.
6. Salix alba, lower epidermis, small enlargement, imprint II.

Microrelief imprint of lower epidermis of Zebrina pendula (large enlargement) prepared with help of various substances: 7 — polyvinyl acetate; 8 — panel lacquer; 9 — fingernail lacquer.

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